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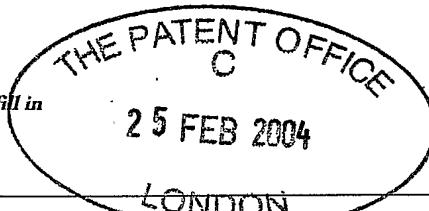
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Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

8036394002

4. Title of the invention

Materials and Methods for Treatment of Allergic Disease

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

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Materials and Methods for Treatment of Allergic Disease

Field of the Invention

5 The present invention relates to materials and methods for the treatment of allergic disease, and particularly although not exclusively to nucleic acids for use in repressing the expression of a STAT6 nucleic acid, peptide, polypeptide or protein.

10

Background to the Invention

15

Asthma is a disease whose incidence is increasing. The cost of available treatments for chronic asthma are rising dramatically and cost-efficient preventative therapeutics are desirable¹.

20

The inflammatory pathology of asthma is predominantly mediated by cytokines which utilise a common transcription factor known as STAT6. STAT6 is critical for allergy development, airway inflammation and asthma (STAT6-deficient animals do not get asthma, even when challenged in a way that induces asthma in normal mice).

25

Drugs that specifically and effectively target STAT6, which resides and operates in the intracellular environment, have proved difficult to develop. To date, no successful drug has been developed that targets STAT6 effectively without causing non-specific side-effects.

30

STAT6

STAT6 is the Signal Transducer and Activator of Transcription 6. To be functional in intact cells, STAT6

has to be phosphorylated. Sequence data for human STAT6 can be accessed from NCBI (www.ncbi.nlm.nih.gov) under accession numbers NP_003144 and U16031.

5 RNAi

RNAi utilises small double-stranded RNA molecules (dsRNA) to target messenger RNA (mRNA), the precursor molecule that cells use to translate the genetic code into 10 functional proteins. During the natural process of RNAi, dsRNA is processed into short-interfering RNA (siRNA) duplexes of 21 nucleotides in length, and it is these molecules which recognise and target homologous (endogenous) mRNA sequences for enzymatic degradation (by 15 complementary base-pair binding), resulting in gene silencing^{3,4}.

The advantages of RNAi over other gene-targeting strategies such as anti-sense oligonucleotides include 20 its relative specificity, its enhanced efficacy (only nanomolar quantities of siRNA are required for efficient gene-silencing), and the fact that siRNA treatment feeds into a natural RNAi pathway that is inherent to all cells.

25 The success of gene-silencing by siRNA can be highly variable depending on the gene target and cell type being targeted.

30 **Summary of the Invention**

The inventors have designed and *in vitro* tested STAT6 siRNA (short interfering RNA). Despite the intrinsic unpredictability of the efficacy of this approach they

obtained specific and highly efficient inhibition of the expression of STAT6 protein in cell types found in lung tissue thus providing effective and specific targeting of STAT6 *in vivo*.

5

The evidence presented herein demonstrates that (anti)STAT6 siRNA, when transferred into cells by cationic lipid-mediated transfer, are indeed functional and efficiently inhibit STAT6 protein expression without 10 obvious side-effects in human cells.

By targeting these siRNA to representative cells from human airways, the inventors have provided the basis of a new therapeutic treatment for patient asthma. In 15 particular, STAT6 siRNA's may be used to treat the local cells of the asthmatic airway via delivery systems such as liposomes or in aerosol form by a standard nebuliser device.

20 The siRNA's provided specifically and efficiently target STAT6 in that they reduce STAT6 gene expression by >90%. Furthermore, cells treated with STAT6-specific siRNA do not express detectable STAT6 protein expression and they do not exhibit phosphorylation of STAT6 protein in 25 response to physiological stimulus with interleukin-4 - in other words, cells treated with individual STAT6 siRNA lose their ability to signal through an intracellular pathway that is heavily implicated in the development of allergic immune responses and associated diseases such as 30 asthma.

For the treatment of asthma, delivery of STAT6 siRNA to the lungs may be achieved by taking advantage of nebulisers which are already standard in conventional

asthma treatments and may take advantage of available commercial formulations (e.g. Smarticles®, Novosom AG, Germany) that may be used to deliver aerosolised siRNA to the lungs.

5

At its most general the present invention relates to nucleic acids and their uses in repressing or silencing the expression of nucleic acids, peptides, polypeptides or protein.

10

More particularly, the present invention relates to the repression of expression of STAT 6 nucleic acid, peptide, polypeptide or protein. Nucleic acids are provided having substantial sequence identity along their length to all or a portion of at least one of a DNA sequence coding for a STAT6 protein or an RNA, e.g. mRNA, encoding a STAT6 protein or a complementary sequence of said DNA or RNA.

15

20

The use of such nucleic acids in the treatment of allergic diseases, e.g. asthma, and in the manufacture of a medicament for the treatment of allergic diseases together with methods of treating allergic diseases are also provided.

25

30

The inventors have also provided methods of repressing or silencing the expression of a STAT6 nucleic acid or protein in vitro and cells in which STAT6 nucleic acid or protein expression is repressed and which may be obtainable by such methods.

In one aspect of the invention a nucleic acid is provided for use in the treatment of allergic disease in an individual.

The nucleic acid preferably represses the expression of STAT6 nucleic acid, polypeptide or protein. Preferably STAT6 nucleic acid or protein function is also repressed.

5

The nucleic acid may be DNA or RNA and may be single or double stranded. Preferably the nucleic acid is an RNA and is double stranded.

10

Preferred nucleic acids include RNA molecules having a sequence of, or complementary to, any of SEQ ID No.s 1-8 and nucleic acids having a sequence identity of at least 60% to one of SEQ ID No.s 1-8 or a complementary sequence therof, and more preferably having at least 70, 80, 85, 90, 95% or 100% sequence identity. DNA molecules encoding RNA's comprising these sequences are also provided.

20

Isolated nucleic acids which may include an RNA molecule having a sequence of, or complementary to, any of SEQ ID No.s 1-8, nucleic acids having a sequence identity of at least 60% to one of SEQ ID No.s 1-8 or a complementary sequence thereof, and more preferably having at least 70, 80, 85, 90, 95 or 100% sequence identity, and DNA molecules encoding RNA's comprising these sequences form another aspect of the invention.

25

In a further aspect of the invention the nucleic acids described are provided for use in the manufacture of a medicament for the treatment of allergic disease, e.g. asthma. Preferably the mechanism of treatment comprises the repression of expression of a STAT6 nucleic acid or protein in vivo.

In yet a further aspect of the invention a method of treating an allergic disease in an individual in need of such treatment is provided. The method may comprise the step of administering to the individual an amount of one or more of the nucleic acids described herein which is effective to treat the symptoms of the allergic disease. The individual may preferably be a human patient in need of treatment.

10 In another aspect of the invention, a method is provided for repressing or silencing the cellular expression of a STAT6 nucleic acid or protein in vitro. The method may comprise the contacting of a cell or cells with a nucleic acid described herein to deliver the nucleic acid to the cell(s). In one arrangement the nucleic acid may be complexed with a carrier, e.g. a lipophilic carrier to assist and/or enhance passage of the nucleic acid across the cell membrane.

15 20 Accordingly, cells may be provided in which the expression of STAT6 nucleic acid or protein is repressed or silenced.

25 30 Suitable cells may be selected from human cells, or alternatively from non-human cells, preferably rat, mouse or other rodent (including cells from any animal in the order Rodentia). Other suitable non-human cells may be e.g., from pig, sheep, non-human primate or other non-human vertebrate organism and/or non-human mammalian cells.

Nucleic acids of the invention may be prepared as part of a pharmaceutical composition comprising a carrier, e.g. a lipophilic carrier or vesicle, or adjuvant in addition to

the nucleic acid. Pharmaceutical compositions and medicaments of the invention may be formulated for oral inhalation or nasal administration, alternatively for parenteral, intravenous or intramuscular administration.

5

For the treatment of asthma or other allergic diseases of the airways, suitable medicaments or therapeutics include those suitable for nasal and/or oral administration (preferably by inhalation) and may be provided as a 10 solution suitable for generation of aerosolised droplets of the medicament for delivery to the airways and lungs by use of an appropriate nebuliser or inhaler.

A STAT6 nucleic acid, polypeptide or protein preferably 15 refers to the nucleic acid encoding a human STAT6 polypeptide or protein or a homologue thereof.

Alternatively, STAT6 may refer to nucleic acid encoding a non-human STAT6 polypeptide or homologue thereof. A non-20 human STAT6 may preferably be selected from any one of a rat, mouse or other rodent (including any animal in the order Rodentia), and may also be selected from a pig, sheep, non-human primate or other non-human vertebrate organism or non-human mammal.

25

STAT6 homologues preferably have at least 60% sequence identity to the STAT6 sequence of the given organism. More preferably the level of sequence identity is at least 70, 80, 90 or 95%.

30

Nucleic acids of the invention may include any of the following double or single stranded RNA sequences.

Sequence ID No.

5'	GCAGGAAGAACUCAAGUUU-3'	1
	3'-CGUCCUUCUUGAGUUCAAA-5'	
5	5'-ACAGUACGUUACUAGCCUU-3'	2
	3'-UGUCAUGCAAUGAUCGGAA-5'	
	5'-GAAUCAGUCAACGUGUUGU-3'	3
	3'-CUUAGUCAGUUGCACAACA-5	
10	5'-AGCACUGGGAGAAAUCAUCA-3'	4
	3'-UCGUGACCUCUUUAGUAGU-5'	
	GCAGGAAGAACUCAAGUUU	5
15	ACAGUACGUUACUAGCCUU	6
	GAAUCAGUCAACGUGUUGU	7
	AGCACUGGGAGAAAUCAUCA	8
20	Furthermore, nucleic acids of the invention may comprise nucleic acid molecules which hybridise with any of SEQ ID No.s 1 to 8 under very high, high or intermediate stringency conditions.	
25	Nucleic acids of the invention may be of any length, but preferred nucleic acids are small and may comprise at least 10 nucleotides and no more than 50 nucleotides. Particularly suitable nucleic acids will have a length in the range 10 to 30 nucleotides and more suitably in the	
30	range 15 to 25 nucleotides. Selected nucleic acid molecules may be any of 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 nucleotides in length.	

Repression and silencing

Nucleic acids of the invention are designed to repress or silence the expression of a target nucleic acid, peptide, 5 polypeptide or protein or to repress a function of such nucleic acid, peptide, polypeptide or protein.

A repression of expression results in a decrease in the quantity of the target. For example, in a given cell the 10 repression of a target by administration of a nucleic acid of the invention results in a decrease in the quantity of the target relative to an untreated cell.

Repression of a function may be the decrease in 15 transcription of an mRNA, or translation of a peptide, polypeptide.

Repression may be partial. Preferred degrees of repression are at least 50%, more preferably one of at 20 least 60, 70, 80, 85 or 90%. A level of repression between 90% and 100% is considered a 'silencing' of expression or function.

Sequence identity

25 Percentage (%) sequence identity is defined as the percentage of nucleic acid residues in a candidate sequence that are identical with residues in the given listed sequence (referred to by the SEQ ID No.) after 30 aligning the sequences and introducing gaps if necessary, to achieve the maximum sequence identity. Sequence identity is preferably calculated over the entire length of the respective sequences.

Where the aligned sequences are of different length, sequence identity of the shorter sequence is determined over the entire length of the longer sequence. For example, where a given sequence comprises 100 nucleotides and the candidate sequence comprises 10 nucleotides, the candidate sequence can only have a maximum identity of 10% to the entire length of the given sequence. This is further illustrated in the following examples:

10 (A)

Given seq: XXXXXXXXXXXXXXX (15 nucleotides)
Comparison seq: XXXXXYYYYYYY (12 nucleotides)

15 % sequence identity = the number of identically matching nucleotides after alignment divided by the total number of nucleotides in the given sequence, i.e. (5 divided by 15) x 100 = 33.3%

(B)

20 Given seq: XXXXXXXXXXXX (10 nucleotides)
Comparison seq: XXXXXYYYYYYZZYZ (15 nucleotides)

25 % sequence identity = number of identical nucleotides after alignment divided by total number of nucleotides in the given sequence, i.e. (5 divided by 10) x 100 = 50%.

Alignment for purposes of determining percent nucleotide sequence identity can be achieved in various ways that are within the skill in the art.

30

Hybridisation stringency

In accordance with the present invention, nucleic acids having an appropriate level of sequence identity may be

identified by using hybridisation and washing conditions of appropriate stringency.

For example, RNA-RNA hybridisations may be performed 5 according to hybridisation methods well known to a person of skill in the art, e.g. the method of Sambrook et al., ("Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 2001).

10 Calculation of the melting temperature (T_m) at a given salt concentration is one method of determining hybridisation stringency. Nucleic acid duplexes of low sequence identity will have a lower T_m than nucleic acid duplexes of higher sequence identity.

15 One of the most accurate derivations of the melting temperature is the nearest-neighbour method. This method is well known to persons of skill in the art, is suitable for calculating the T_m of short nucleic acids and takes 20 into account the actual sequence of the oligonucleotides as well as salt concentration and nucleic acid concentration.

25 The nearest-neighbour equation for both DNA and RNA based oligonucleotides is:

$$T_m = \left[\frac{[1000\Delta H^\circ]}{A + \Delta S^\circ + R \ln(C_t / 4)} \right] - 273.15 + 16.6 \log[Na^+]$$

30 where ΔH° (Kcal/mol) is the sum of the nearest-neighbour enthalpy changes for hybrids, A is a constant (-10.8) correcting for helix initiation, ΔS° is the sum of the nearest neighbour entropy changes, R is the Gas Constant (1.99 cal $K^{-1}mol^{-1}$) and C_t is the molar concentration of

the oligonucleotide. ΔH° and ΔS° values for both DNA and RNA nearest neighbour bases are publicly available (e.g. from Genosys Biotechnologies Inc.).

5 In general for RNA-RNA hybridisations under very high stringency conditions, the melting temperature of RNA duplexes of 100% sequence identity would be expected to be approximately greater than or equal to 60°C, although the actual T_m for any given duplex requires empirical 10 calculation.

Accordingly, nucleotide sequences can be categorised by an ability to hybridise under different hybridisation and washing stringency conditions which can be appropriately 15 selected using the above equation or by other similar methods known to persons skilled in the art.

Sequences exhibiting 95-100% sequence identity are considered to hybridise under very high stringency 20 conditions, sequences exhibiting 85-95% identity are considered to hybridise under high stringency conditions, sequences exhibiting 70-85% identity are considered to hybridise under intermediate stringency conditions, sequences exhibiting 60-70% identity are considered to 25 hybridise under low stringency conditions and sequences exhibiting 50-60% identity are considered to hybridise under very low stringency conditions.

The invention includes the combination of the aspects and 30 preferred features described except where such a combination is clearly impermissible or expressly avoided.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All 5 documents mentioned in this text are incorporated herein by reference.

Brief Description of the Figures

10 **Figure 1** *Design of siRNA targeting STAT6.*
Targeted DNA sequences encoding parts of the STAT6 mRNA and the corresponding duplex structure of the prepared siRNA are shown.

15 **Figure 2** *Inhibition of STAT6 Expression by RNAi.*

A549 cells were treated with individual siRNA at a final concentration of 100 nM.

20 (A). 60 hours after treatment cellular proteins were harvested, equal amounts (10 µg) separated by molecular weight using electrophoresis and immobilised onto synthetic membranes (Western Blotting). The presence of STAT6 protein (120 kDa) was then detected using a polyclonal αSTAT6 antibody (Santa Cruz Biotechnology, 25 Ca., USA) where the amount of STAT6 expression in each sample correlates with band density. Cells treated with STAT6(1) siRNA had no detectable expression of STAT6 protein (no visible band). In lanes 2 & 3 (STAT6 (2)-, STAT6(3)-treated) STAT6 protein bands are barely 30 detectable, indicating significant inhibition (>95%). STAT6(4) siRNA was the least efficient although this siRNA still inhibited STAT6 expression by 90%. In contrast, control scrambled siRNA (scGAPDH, lane 6) had

no effect on STAT6 expression. Similarly, STAT6 siRNA had no effect on GAPDH expression which is readily detectable as a 37 kDa protein band (using a GAPDH-specific antibody).

5

(B). STAT6 gene expression (mRNA production) in siRNA-treated cells was measured by real-time RT-PCR, allowing absolute quantification of gene expression. By comparing the amount of STAT6 expression to the housekeeping gene GAPDH (i.e. the ratio of STAT6/GAPDH expression: y-axis) the specific effects of siRNA can be measured. As shown, STAT6 siRNA (1-4) inhibit STAT6 mRNA by $\geq 90\%$. In contrast, cells treated with scGAPDH siRNA do not exhibit any reduction in STAT6 mRNA expression, indicating that the transfection procedure itself does not inhibit the STAT6 gene.

Figure 3 RNAi of STAT6 leads to loss of STAT6 function. To measure STAT6 activity A549 cells were cultured in the presence (right histogram) or absence (left histogram) of IL-4 (1 ng/ml) for 30 minutes prior to staining with anti-phospho-STAT6: Alexa fluor-647 labelled antibody (BD PharMingen, Oxford, UK). This antibody only recognises STAT6 molecules that are phosphorylated on tyrosine residue 641. After staining procedures, fluorescence in cells was measured on a flow cytometer. In the histograms above, the amount of bound antibody is indicated by the relative amount of detectable fluorescence in individual cells (x-axis). The amount of fluorescence that is detectable above background levels is indicated in the gated region marked P2. As shown, IL-4 was capable of activating STAT6 in cells as indicated by the increase in fluorescence (top row, 35.3% versus 5.1% background in unstimulated cells). In

contrast, when cells were treated with STAT6-specific siRNA, the ability of IL-4 to activate STAT6 was completely abolished (bottom row, 11.2% fluorescence in both stimulated and unstimulated cells)

5

Detailed Description of the Best Mode of the Invention

Specific details of the best mode contemplated by the inventors for carrying out the invention are set forth below, by way of example. It will be apparent to one skilled in the art that the present invention may be practiced without limitation to these specific details.

STAT6 siRNA

15

Sequences within the coding region of STAT6 mRNA (GenBank, U16031) only were selected for targeting by siRNA. Regions near the start codon (within 75 bases) were avoided as they may contain regulatory protein binding sites.

20

To ensure specificity, selected target sites were compared by BLAST® (NCBI) search for homology with other known coding sequences. Target sequences were also selected on the basis of having a GC content < 40% and beginning with AA to allow thymidine overhangs (tt) in the subsequent siRNA (Fig.1).

25

Pure STAT6 siRNA duplexes were chemically synthesised according to the inventors design by Ambion Inc. (Austin, TX, USA) and supplied as dried RNA oligonucleotide. Reconstituted siRNA were subsequently employed in cell treatment experiments.

30

Targeting of STAT6 Gene Expression by STAT6 siRNA

STAT6-expressing lung epithelial cells (A549) were treated with individual STAT6 siRNA and their ability to subsequently inhibit STAT6 expression determined by 5 measuring both STAT6 mRNA and STAT6 protein expression (Fig. 2). To ensure efficient cellular targeting, siRNA were complexed with a commercially available cationic lipid reagent (LipofectamineTM, Invitrogen) and transfected into cells.

10

STAT6 siRNA were validated against commercially available α GAPDH ('housekeeping' gene) siRNA and scrambled GAPDH siRNA with no known homology to human mRNA sequences (Ambion, Inc.) i.e. positive and negative controls 15 respectively. In these experiments STAT6 siRNA duplexes were shown to inhibit the expression of both STAT6 mRNA and STAT6 protein expression in treated cells. Furthermore, this suppression was STAT6-specific in that the expression of non-related housekeeping genes such as 20 GAPDH, were not affected.

STAT6 Function is Abolished by STAT6 siRNA Treatment

In order for RNAi to be a successful therapeutic it is 25 essential that the targeting of genes leads to loss of protein function within treated cells. Therefore, in addition to measuring STAT6 expression (as shown above), we determined the effects of siRNA treatment on STAT6 activity within cells. As STAT6 protein has to become 30 phosphorylated within cells in order for it to mediate its effects, we employed an assay that directly measures the amount of phosphorylated STAT6 within intact cells. This assay utilises an anti-phospho-STAT6 antibody (BD-PharMingen) that fluorescently labels cells expressing

the phosphorylated STAT6 protein. The amount of detectable fluorescence in IL-4 treated cells (measured by flow cytometry) is directly related to the amount of phosphorylated STAT6 (Fig. 3).

5

To activate STAT6, cells were stimulated with interleukin-4 (IL-4), a chemical messenger that is produced during allergic responses and naturally activates STAT6 in cells. Using this assay we were able 10 to unequivocally demonstrate that RNAi of STAT6 leads to elimination of STAT6 function (phosphorylation) in cells.

A 90% inhibition of gene expression does not necessarily correlate with complete loss of STAT6 protein expression 15 and therefore by extrapolation, its function within cells. Accordingly, the antibody staining experiments (flow cytometry - Fig.3) were performed. These results show that STAT6 siRNA treatment leads to loss of STAT6 function. STAT6 protein expression was inhibited 20 following siRNA treatment (as demonstrated by Western Blotting) and this deficiency appears to be absolute in that STAT6-phosphorylation in response to interleukin-4 stimulation could not be detected by flow cytometry.

25

Discussion

The results described show that by designing siRNA specific to STAT6 effectively inhibition of STAT6 gene and protein expression can be achieved in cell types that 30 are relevant to asthma. Importantly, the results also demonstrate that treatment of cells with STAT6 siRNA leads to the abolition of STAT6 function upon stimulation with physiological stimuli.

As STAT6 is known to be a central mediator of many of the dysregulated processes that take place in asthma development, the targeting of this gene by this approach provides a route of unique therapy for asthmatic disease.

5

Accordingly, an STAT6 siRNA based treatment for allergic disease is provided which, in the case of asthma, operates by selectively down-regulating STAT6 expression to inhibit the asthma-inducing effects of STAT6 in patients.

10

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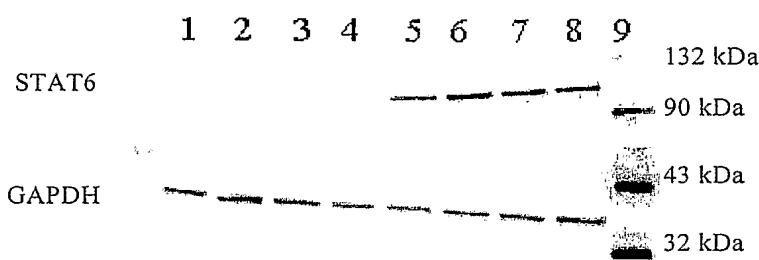
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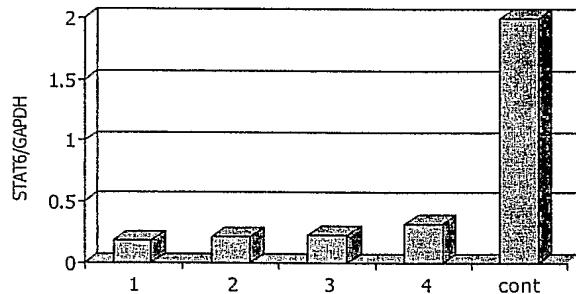
Fig. 1 Design of siRNA Targeting STAT6

DNA sequence encoding mRNA	siRNA Duplex Structure
STAT6(1): AAGCAGGAAGAACCTCAAGTTT	5'-GCAGGAAGAACUCAAGUUUtt-3' 3'-ttCGUCCUUCUUGAGUUCAAA-5'
STAT6(2): AACACAGTACGTTACTAGCCTT	5'-ACAGUACGUUACUAGCCUtt-3' 3'-ttUGUCAUGCAAUGAUCGGAA-5'
STAT6(3): AAGAACATCAGTCAACGTGTTGT	5'-GAAUCAGUCAACGUGUUGUtt-3' 3'-ttCUUAGUCAGUUGCACAACA-5'
STAT6(4): AAAGCACTGGAGAAATCATCA	5'-AGCACUGGAGAAAUCAUCAUCAtt-3' 3'-ttUCGUGACCUCUUAGUAGU-5'



Fig. 2 Inhibition of STAT6 Expression by RNAi**A**

1= STAT6(1) siRNA
 2= STAT6(2) siRNA
 3= STAT6(3) siRNA
 4= STAT6(4) siRNA
 5= GAPDH siRNA
 6= scGAPDH siRNA
 7= untreated cells
 8= untreated + IL-4
 9= molecular weight markers

B

1= STAT6(1) siRNA
 2= STAT6(2) siRNA
 3= STAT6(3) siRNA
 4= STAT6(4) siRNA
 cont = scGAPDH siRNA



Fig. 3 RNAi of STAT6 Leads to Loss of STAT6 Function

